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# Construction and Characterization of Mutant Water-Soluble PQQ Glucose Dehydrogenases with Altered $K_m$ Values—Site-Directed Mutagenesis Studies on the Putative Active Site

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**Based on a PCR mutant enzyme of water-soluble glucose dehydrogenase-harboring pyrroloquinoline quinone as the prosthetic group, PQQGDH-B, a site-directed mutagenesis study was carried out. The substitution of Glu277 residue with Gly resulted in a decrease in the $K_m$ value for glucose and altered the substrate specificity profile, compared with the wild-type enzyme. Mutational analyses on the neighboring amino acid residues of Glu277 were also carried out and constructed Asp275Glu, Asp276Glu, Ile278Phe, and Asn279His. Considering that Asp275Glu, Asp276Glu and also Glu277Gly showed drastic decreases in EDTA tolerance, this region may construct a PQQGDH-B putative active site, such as a binding site for $Ca^{2+}$, which is responsible for the binding PQQ. A series of Glu277 variants, Glu277 substituted by Ala, Val, Asp, Asn, His, Gln, or to Lys, was constructed and they all showed decreased $K_m$ values and altered substrate specificity profiles. Among them, Glu277Lys showed similar thermal stability with the wild-type enzyme, but its catalytic efficiency increased significantly, compared with the wild-type enzyme. The potential applications of Glu277Lys in analytical use are also discussed.** © 1999 Academic Press

*Key Words:* **pyrroloquinoline quinone (PQQ); glucose dehydrogenase; s-GDH; catalytic activity; biosensor.**

Considerable attention has been devoted to the application of glucose dehydrogenases (GDHs) harboring pyrroloquinoline quinone (PQQ) as their prosthetic group for the glucose enzyme sensor constituent. Since PQQGDHs do not utilize oxygen as electron acceptors during the oxidation of glucose, they are considered to be ideal constituent for electron-mediator type glucose sensor (1–7). Although PQQGDHs have such superior properties, further improvement of the enzymatic properties is being required, considering and comparing with those of the most popular and major enzymes utilized for glucose sensors, glucose oxidase, e.g. substrate specificity and operational stability. In order to construct an ideal PQQGDH with modified enzymatic properties by protein engineering, further structural information together with the rational mutagenesis studies are essential. Two types of PQQGDHs have been reported; the membrane-binding single peptide PQQGDH (PQQGDH-A or m-GDH), and the water-soluble dimeric PQQGDH (PQQGDH-B or s-GDH). The putative active site and amino acid residue responsible for substrate recognition of PQQGDH-A, have been reported, based on both the experimental analyses (8–16) and structural prediction(17). However, there have not yet been reported any mutational analyses for PQQGDH-Bs. The lack of information on the structure–function relationship limited the improvement in the enzymatic properties of PQQGDH-B via the protein engineering approach.

This paper reports on site-directed mutagenesis studies of PQQGDH-B putative active site, based on the enzymatic properties of PCR mutant of this enzyme. The mutant enzyme found in our laboratory, designated as No. 87, contained 8 amino acid substitutions; Thr71Ala, Lys264Glu, Leu317Ser, Thr331Ala, Arg407Leu, Ser415Gly, Lys455Ile and Glu277Gly. The No.87 mutant showed a decreased $K_m$ value and also decreased EDTA tolerance and thermal stability compared with those of the wild type. Among these 8 substitutions, we investigated the substitution of Lys264 for Glu, Arg407 for Leu and Glu277 for Gly substitutions (the results were not shown), and found that Glu277Gly showed the similar properties to the No. 87 mutation. Based on

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| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wild type | | 3'- G | AGA | CTG | CTA | CTT | TAA | TTG | GAG | -5' | | |
| | 272 | 273 | 274 | 275 | 276 | 277 | 278 | 279 | 280 | 281 | 282 | |
| | Pro | Asn | Ser | Asp | Asp | Glu | Ile | Asn | Leu | Ile | Val | |
| Glu277Gly | | 3'- G | AGA | CTG | CTA | CCT Gly | TAA | TTG | GAG | -5' | | |
| Glu277Ala | | 3'- G | AGA | CTG | CTA | CGT Ala | TAA | TTG | GAG | -5' | | |
| Glu277Val | 3'- GT | TTG | AGA | CTG | CTA | CAT Val | TAA | TTG | GAG | TAA | C -5' | |
| Glu277Asp | | 3'- G | AGA | CTG | CTA | TTT Asp | TAA | TTG | GAG | -5' | | |
| Glu277Lys | | 3'- G | AGA | CTG | CTA | CTA Lys | TAA | TTG | GAG | -5' | | |
| Glu277His | 3'- GT | TTG | AGA | CTG | CTA | GTG His | TAA | TTG | GAG | TAA | C -5' | |
| Glu277Asn | 3'- GT | TTG | AGA | CTG | CTA | TTG Asn | TAA | TTG | GAG | TAA | C -5' | |
| Glu277Gln | | 3'- G | AGA | CTG | CTA | GTT Gln | TAA | TTG | GAG | -5' | | |
| Asp275Glu | 3'-GGT | TTG | AGA | CTT Glu | CTA | CTT | TAA | TTG | GAG | -5' | | |
| Asp276Glu | 3'- GT | TTG | AGA | CTG | CTT Glu | CTT | TAA | TTG | GAG | -5' | | |
| Ile278Phe | | 3'- G | AGA | CTG | CTA | CTT | AAG Phe | TTG | GAG | TAA | C -5' | |
| Asn279His | | 3'- TTG | AGA | CTG | CTA | CTT | TAA | GTG His | GAG | TAA | CAG -5' | |

**FIG. 1.** The oligonucleotide primers and corresponding peptide sequences of PQQGDH-B variants.

these findings, we developed a series of Glu277 variants, and also introduced mutation into its neighbors, Asp275, Asp276, Ile278 and Asn279. $K_m$ values, substrate specificity, the EDTA tolerance and thermal stability of these mutants were investigated.

## MATERIALS AND METHODS

*1. Bacterial strains and plasmid.* *E. coli* PP2418, of which the PQQGDH structural gene was disrupted by insertion mutagenesis (18), was used as the host strain for the expression for each PQQGDH-B. *Acinetobacter calcoaceticus* LMD79.41 was obtained from the Netherlands Culture Collection (NCC), as a source of the genomic fragment containing the PQQGDH-B structural gene. *E. coli* BMH71-18 *mutS* and *E. coli* MV1184 were used to construct mutations by site-directed mutagenesis. All of the PQQGDH structural genes were inserted into the multicloning site of the expression vector, pTrc99A (Pharmacia, Sweden).

*2. Genetic manipulation.* Error-prone PCR (19) amplification of the PQQGDH-B structural gene was carried out using a genomic gene extracted from *A. calcoaceticus* LMD79.41 as the template, and following one set of oligonucleotide as the primers. Forward, 5'-GGCCATGGATAAACATTTATTGGCTAAAATTGCTTTAT-3'; reverse, 5'-GGAAGCTTTTACTTAGCCTTATAGGTGAACTTAATGAG-3'.

Then amplified fragment was digested by *NcoI*–*Hin*dIII, and inserted in the multi-cloning site of pTrc99A. The sequence of inserted gene fragments was analyzed with an automated DNA sequencer (PE Applied Biosystems 310 Genetic Analyzer, California).

Site-directed mutagenesis was carried out using the gene fragment (1.2 kbp) of the wild-type PQQGDH-B structural gene obtained by PCR amplification, which was inserted in pTrc99A, designated as pGB (20). The *KpnI*–*Hin*dIII fragment was obtained from pGB, and the resultant 1.2kbp fragment was inserted in the linearized pKF18k (Takara, Japan), after digestion by *KpnI* and *Hin*dIII. The oligonucleotide primers used for the site-directed mutagenesis are summarized in Fig. 1. The position of amino acid residues are defined relative to the initiator methionine residue.

Site-directed mutagenesis was performed according to the instruction manual in Mutan-Express Km kit (Takara, Japan). The nucleotide sequence of mutation was confirmed by an automated DNA sequencer. This obtained mutated region was then digested with *KpnI* and *Hin*dIII, and the fragment was substituted into the corresponding region of pGB. In this way, expression vectors containing mutated PQQGDH-B were constructed.

*Enzyme preparation and enzyme assay.* Wild-type and mutant PQQGDH-B samples were prepared according to previous studies (20–23) with slight modification. A crude enzyme samples were prepared by ultrasound sonication (VC 100, BIOMIC, Connecticut), following the dialysis with 10 mM MOPS-NaOH (pH 7.0). Crude enzyme sample of Glu277Lys was subjected to a CM-Toyopearl 650 M cation exchange column (Tosoh, Japan) and subsequently a hydroxyapatite column (Merck, Germany) as described previously for wild-type enzyme preparation (20).

GDH activity was measured using 0.6 mM phenazine methosulfate (PMS) and 0.06 mM 2,6-dichrolophinolindophenol (DCIP) after incubation in 10 mM MOPS-NaOH (pH 7.0) containing 1 mM $CaCl_2$ and 1 $\mu$M PQQ for 30 minute. Each activity at 100 mM glucose was calculated by monitoring the decrease in absorbance of DCIP at 600 nm.

The thermal stability of the enzymes was determined using crude enzyme preparations. Since the initial time course for thermal inactivaton at 55°C was describable by first-order kinetics, from the linear regression of the logarithmic for residual activity against time, the thermal stability of each variant was expressed with a half-life ($t_{1/2}$) at 55°C.

EDTA tolerance for all of mutants was determined using crude enzyme samples, by incubating them in 10 mM MOPS-NaOH (pH 7.0) containing 5 mM EDTA, and samples were taken periodically, with their residual activity being expressed.

TABLE 1
Substrate Specificity and Half-Life Time at 55°C of PQQGDH-Bs

| | Glu277 (wild type) | No. 87 | Glu277Gly | Glu277Ala | Glu277Asp | Gly277Lys | Glu277His | Glu277Asn | Glu277Gln | Glu277Val | Asp275Glu | Asp276Glu | Ile278Phe | Asn279Ile |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $K_m$ value (mM) | 28.0 | 2.4 | 0.3 | 1.5 | 7.4 | 8.9 | 7.7 | 1.2 | 4.3 | 2.5 | 24.0 | 24.0 | 7.0 | 15.7 |
| $t_{1/2}$ at 55°C (min) | 10 | 5 | <2 | <2 | <2 | 10 | <2 | <2 | 4 | <2 | <2 | <2 | 25 | 4 |
| Glucose | 100 (%) | 100 (%) | 100 (%) | 100 (%) | 100 (%) | 100 (%) | 100 (%) | 100 (%) | 100 (%) | 100 (%) | 100 (%) | 100 (%) | 100 (%) | 100 (%) |
| 2-Deoxy-D-glucose | 4 | 18 | 15 | 11 | 9 | 7 | 3 | 8 | 10 | 8 | 6 | 13 | 4 | 2 |
| Mannose | 13 | 34 | 38 | 33 | 19 | 18 | 11 | 29 | 31 | 28 | 11 | 19 | 14 | 6 |
| Allose | 47 | 124 | 180 | 142 | 68 | 116 | 67 | 148 | 134 | 150 | 44 | 54 | 54 | 48 |
| 3-O-Methyl-D-glucose | 81 | 99 | 113 | 91 | 38 | 94 | 45 | 91 | 80 | 101 | 45 | 57 | 47 | 50 |
| Galactose | 11 | 38 | 39 | 46 | 21 | 28 | 12 | 43 | 35 | 45 | 12 | 28 | 15 | 13 |
| Xylose | 7 | 25 | 28 | 25 | 15 | 18 | 9 | 18 | 25 | 18 | 7 | 20 | 12 | 8 |
| Lactose | 61 | 95 | 100 | 83 | 87 | 79 | 57 | 86 | 68 | 114 | 52 | 75 | 64 | 64 |
| Maltose | 61 | 58 | 52 | 39 | 73 | 74 | 39 | 59 | 54 | 85 | 44 | 66 | 49 | 61 |

*Note.* Each enzyme activity was measured at 20 mM substrate concentration and compared with glucose as the control.

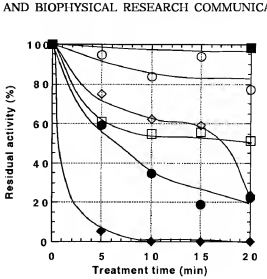


**FIG. 2.** EDTA tolerance of PQQGDH-Bs. In the presence of 5 mM EDTA at 25°C, time courses of residual activity were measured. The initial activities showed in the absence of EDTA were used as a control. ○, wild-type; ●, No. 87; □, Glu277Gly; ■, Glu277Lys; ◇, Asp275Glu; ◆, Asp276Glu.

## RESULTS

### *1. Site-Directed muTagenesis Studies on Asp275, Asp276, Glu277, Ile278, and on Asn279*

Table 1 summarizes the enzymatic properties of mutant PQQGDHs constructed in this study, using crude enzyme preparation.

The random mutant, No. 87 showed significant decrease in the $K_m$ value for glucose, compared with wild-type. The substrate specificity profile for No. 87 was different from that of the wild-type enzyme. The mutant showed the altered substrate specificity toward 2-deoxy-D-glucose, mannose, allose, galactose and for xylose had up to a 2 to 3-fold increase in the relative activity against glucose, compared with the wild-type enzyme. In addition to the alteration of the $K_m$ value and substrate specificity, the No. 87 mutation also resulted in the decrease in the EDTA tolerance (Fig. 2) and thermal stability (Table 1).

Among the 8 amino acid substitutions of the No. 87 mutant, we confirmed that the Glu277 substitution for Gly was responsible for the characteristic property of the No. 87 mutant. Glu277Gly also showed a decreased $K_m$ value (0.3 mM) and a similar substrate specificity profile as No. 87. Glu277Gly also showed a significant decrease in both EDTA tolerance and thermal stability.

On the basis of this finding, we also introduced mutations in the surrounding amino acid residues of Glu277, Asp275, Asp276, Ile278 and Asn279.

Among the mutations introduced in the neighboring of Glu277 and Ile278, there was also a decrease in the $K_m$ value, but this mutation did not have an impact on the alteration in the substrate specificity (Table 1).

Asp275Glu and Asp276Glu resulted in a drastic decrease in the EDTA tolerance (Fig. 2) and a decrease in

**TABLE 2**

Kinetic Parameters of Wild Type and Glu277Lys for Various Substrates

| | Wild type | | | | Glu277Lys | | | |
|---|---|---|---|---|---|---|---|---|
| | $K_m$ (mM) | $V_{max}$ (U mg$^{-1}$) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) | $K_m$ (mM) | $V_{max}$ (U mg$^{-1}$) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) |
| Glucose | 26.8 | 4104 | 3436 | 128 | 8.8 | 3668 | 3071 | 349 |
| 2-Deoxy-D-glucose | 90.0 | 395 | 331 | 4 | 88.0 | 1269 | 1063 | 12 |
| Mannose | 22.0 | 319 | 267 | 12 | 22.0 | 1028 | 861 | 39 |
| Allose | 29.0 | 1723 | 1443 | 50 | 21.0 | 5450 | 4563 | 287 |
| 3-*O*-m-D-Glucose | 46.0 | 1729 | 1448 | 31 | 27.0 | 3820 | 3198 | 118 |
| Galactose | 6.8 | 144 | 121 | 18 | 6.8 | 753 | 630 | 78 |
| Xylose | 14.3 | 240 | 201 | 7 | 34.0 | 810 | 678 | 20 |
| Lactose | 14.3 | 799 | 669 | 47 | 7.5 | 2144 | 1795 | 239 |
| Maltose | 30.9 | 937 | 785 | 25 | 14.3 | 1212 | 1015 | 71 |

the thermal stability (Table 1). The Ile278Phe substitution resulted in an increase in the thermal stability (Table 1) but did not have as much of an effect on EDTA tolerance, as the Asn279His substitution did.

### *2. Construction and Characterization of Glu277 Variants*

We further constructed a series of Glu277 variants, Glu277Ala, Glu277Val, Glu277Asp, Glu277Asn, Glu277His, Glu277Gln and Glu277Lys. All the Glu277 variants showed decreased $K_m$ values for glucose and altered substrate specificity profile compared with wild-type PQQGDH-B (Table 1). These observation confirmed that Glu277 has a crucial role in determining the $K_m$ values and consequently for substrate specificity. Except for Glu277Gly, all of the Glu277 variants showed similar EDTA tolerance to wild-type PQQGDH-B. The thermal stability for Glu277 variants was less than 20% of the wild-type except Glu277Lys.

Considering that Glu277Lys had a relatively high thermal stability, and in contrast, the other Glu277 variants were very unstable enzymes not suitable for further purification procedures and not practical targets for future application studies, we purified Glu277Lys and analyzed its kinetic parameters. The results are summarized in Table 2. The $K_m$ value for glucose of purified Glu277Lys was 8.8 mM and was about 40% of that of wild-type PQQGDH-B. The decrease in the $K_m$ value was also observed for another substrate, such as for allose (29.0 mM to 21.0 mM), 3-*O*-methyl-D-glucose (46.0 mM to 27.0 mM), lactose (14.3 mM to 7.5 mM) and for maltose (30.9 mM to 14.3 mM), respectively. In contrast, the $K_m$ values for mannose and for galactose were not altered. The wild-type PQQGDH-B showed the highest specific activity for glucose, however, Glu277Lys showed the highest specific activity for allose (5450 U mg$^{-1}$), the second-highest for 3-*O*-methyl-D-glucose (3820 U mg$^{-1}$), and the specific activity for glucose (3668 U mg$^{-1}$) was the third. Consequently, the catalytic efficiency ($k_{cat}/K_m$) of Glu277Lys, 349 $s^{-1}mM^{-1}$, was about three-fold higher than that of wild type (128 $s^{-1}mM^{-1}$).

## DISCUSSION

In this paper, we have constructed and characterized a series of PQQGDH-B mutants, on the basis of finding of enzymatic properties for a PCR random mutant, No. 87, which harbors 8 amino acid substitution compared with wild-type enzyme.

Considering that all the constructed Glu277 variants showed decreased $K_m$ values and altered substrate specificity profiles, Glu277 has a crucial role in determining its affinity toward the substrate.

It was also notable that the No. 87 mutant, as well as Glu277Gly, Asp275Glu and Asp276Glu, did not show EDTA tolerance, and were readily inactivated in the presence of 5 mM EDTA (Fig. 2). It has been reported that PQQGDH-B is a dimeric enzyme, containing the dual roles for $Ca^{2+}$ (25), one for the maintenance of dimeric enzyme and the other for the binding of PQQ onto the active site. Since the mutation at Glu277 significantly affected in the catalytic properties of PQQGDH-B, Asp275, Asp276 and Glu277 may construct a putative active site involving the $Ca^{2+}$ binding sites.

The kinetic parameters of Glu277Lys showed that this mutation resulted in the increase in the catalytic efficiency of PQQGDH-B (Table 2). Such observation was not limited for glucose, but the catalytic efficiency for all the substrate tested here significantly increased.

Recently, a variety of methods have been proposed to non- or semi-invasive monitoring of blood-glucose levels. As Glu277Lys showed increased specific activity with a low $K_m$ value (Table 2), and also retained similar EDTA tolerance and thermal stability as wild-type PQQGDH-B, this variant has great potential in the application for highly-sensitive glucose monitoring. This high sensitivity of enzymes indicated that the

potential application of this enzyme in the measurement of the glucose concentration present in extracted interstitial fluid (ISF), which is expected to be a principle for the practical semi-invasive glucose monitoring system. The sampling volume of ISF is tiny and also the glucose concentration of ISF is far lower than blood, the use of such a sensitive enzyme as Glu277Lys should be advantageous. The sampling procedure for ISF is painless, therefore, the combination of an enzyme glucose sensor employing Glu277Lys and adequate ISF sampling device will enable us to construct a semi-invasive blood-glucose monitoring system.

In conclusion, this paper demonstrated that Glu277 plays a crucial role in the catalytic activity of PQQGDH-B, especially in dominating the $K_m$ value. Considering the mutation in resulted in not only in the alteration of the specific activities and $K_m$ values but also affected in the EDTA tolerance, the indicator for co-factor binding stability, Glu277 and its neighboring amino acid residue may construct the active site of PQQGDH-B. These observations indicate, to a significant degree, the modification of enzymatic properties of PQQGDH-B together with further structural information of this enzyme.

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